Short Communication

A Reproducibility Study for a Fluoride Assay in Bone

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Abstract

Osteosarcoma is a rare malignancy of largely unknown etiology. Although there is no consistent evidence for an association between fluoridation and cancer, some concerns remain about osteosarcoma. As part of the design of a collaborative study, bone samples were collected to allow for an evaluation of the association between osteosarcoma risk and individual fluoride exposure measured by levels of fluoride in bone. In this report, we provide the results of pilot experiments to consider issues that arose during the study design and to assess the reliability of the bone assays. Correlations of fluoride levels between normal bone near the affected area and iliac crest bone were strong and positive. The day-to-day laboratory analysis of fluoride in human and deer jaw bone yielded acceptable average coefficients of variation below 10% and an overall estimate of 5%. The intraclass correlation (ICC) is of particular importance to epidemiologists because it indicates the effect of measurement error on study results. Here, the estimated ICC is 0.86, and the estimated downward bias is only 14%. Hence, the ICC is strong enough so that the estimates of the relative risk will suffer little attenuation from lab measurements. (Cancer Epidemiol Biomarkers Prev 2006;15(5):1035–7)

Introduction

Periodically, antifluoridation groups have raised concerns about possible adverse effects of water fluoridation on chronic disease rates, especially cancer. In general, descriptive epidemiologic studies have not found a significant increase in human cancer incidence or mortality due to the implementation of community water fluoridation (1-3). In a large bioassay, the National Toxicology Program found a small nonsignificant number of rare osteosarcoma in male rats fed doses of highly fluoridated water with no evidence or risks in female rats or male or female mice (4). In response, the National Cancer Institute evaluated patterns of osteosarcoma incidence over time (5). This revealed some age- and sex-specific increases over time in osteosarcoma rates that were more prominent in fluoridated than in nonfluoridated areas. However, further analysis revealed that these increases were unrelated to the timing of fluoridation. Thus, whereas there is no consistent evidence for an association between fluoridation and cancer, some concerns remain about osteosarcoma.

Osteosarcoma is a rare malignancy of largely unknown etiology (6). The Bone Disease and Injury Study of Osteosarcoma was begun as a collaboration effort between National Cancer Institute and the Harvard School of Dental Medicine to search for the causes of osteosarcoma. This study is an extension and expansion of a previous study of fluoride and osteosarcoma conducted by Harvard School of Dental Medicine. The collaborative study was conducted from 1993 to 2000 in the same 10 participating U.S. hospitals included in the prior study but used a prospective hospital-based case-control design to study incident cases. As part of the design of the collaborative study, bone samples were collected to allow for an evaluation of the association between osteosarcoma risk and individual fluoride exposure measured by levels of fluoride in bone. In this report, we provide the results of pilot experiments to consider issues that arose during the study design and to assess the reliability of the bone assays.

Materials and Methods

Experimental Design. The pilot used specimens obtained from study subjects who were ineligible (n = 20 subjects). Although the samples were not selected at random, we know of no reason that they should not be considered representative. Bone specimens were ashed and randomized before being sent to the laboratory.

Type of Bone Specimen. Unaffected frozen bone specimens adjacent to the tumor site were collected from all study subjects. For five pilot subjects, iliac crest bone samples had also been collected and stored as frozen. A paired t test and both Spearman and Pearson correlations were used to compare the fluoride measurements from the frozen unaffected bone with frozen iliac crest samples.

Method of Storage. Bone specimens from the margins of tissue removed from study subjects during surgery were collected and stored as frozen and formalin (10%) fixed samples. The fluoride measurements from samples using the two methods of specimen storage were compared using a paired t test and both Spearman and Pearson correlations.

Measurement Reproducibility. The correlation between measurements on different samples from a given individual is the intraclass correlation (ICC). The ICC is of importance to the epidemiologist because it indicates the effect of measurement error on study results (7, 8).

Measurements were analyzed on the logarithmic scale to reduce the dependence of the SD of the response on the mean. Samples included those described above along with a few

Received 10/12/05; revised 2/23/06; accepted 3/9/06.

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doi:10.1158/1055-9965.EPI-05-0796

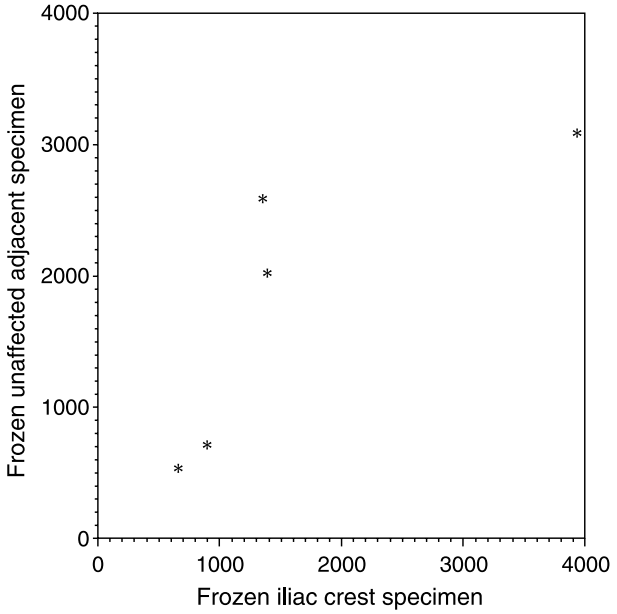

Figure 1. Fluoride measurement using frozen unaffected adjacent samples compared with those based on frozen samples from the iliac crest for five subjects.

single samples. Let z_{ijk} denote the fluoride measurement for subject i, and the statistical model is

$$\mathrm{Log_e}(z_{ijk}) = \mu + T_j + S_k + a_i + \varepsilon_{ijk}, \quad \text{(A)}$$

where T_j and S_k are the fixed effects for bone type (j = 1, 2) and storage (k = 1, 2); a_i is the random effect for subjects, and ε_{ijk} is the random error.

The estimated ICC between measurements from two bone samples of the same type of bone obtained from a single subject and stored in the same manner is

$$\hat{\mathrm{ICC}} = 100\hat{\sigma}_a^2/(\hat{\sigma}_a^2 + \hat{\sigma}^2) \quad \text{(B)}$$

Note that σ^2 includes laboratory assay variability along with the variability associated with ashing and with different bone samples from a subject.

Assay variability. The measure of assay variability commonly used by labs is the coefficient of variation (CV). Sufficient bone sample was collected from two subjects for estimation of the assay variability. The bone sample from each subject was ashed before aliquoting. The lab received five batches of ashed samples, with one batch to be assayed at the beginning of each of four consecutive weeks. Each batch contained two aliquots from each of the subjects. Each aliquot was then assayed in duplicate.

A nested component of variance analysis was done with logarithmically transformed measurements. The CV expressed as a percentage is then estimated by

$$\hat{CV} = 100(\hat{\sigma}_b^2 + \hat{\sigma}_c^2 + \hat{\sigma}_\varepsilon^2/2)^{1/2} \quad \text{(C)}$$

where σ_b^2, σ_c^2, and σ_ε^2 are the variance components for day, aliquot, and error, respectively. Note that σ^2 in this model does not include variability associated with ashing or bone sampling as was the case in Eq. B.

Because sufficient human bone samples were available for only two subjects, deer jaw bone samples from three deer were also obtained and ashed, and aliquots were sent to the lab as part of the assay variability study.

Laboratory Methods. Fluoride analyses of bone ash were carried out after overnight hexamethyldisiloxane (Dow Chemicals, Buffalo Grove, MI) facilitated diffusion using the ion-specific electrode (Model 9409, Orion Research, Beverly, MA) and a miniature calomel reference electrode coupled to a potentiometer (Model 720A, Orion Research). The method used was that developed by Taves (1968) as modified by Whitford (9, 10).

After having been ashed in porcelain crucibles at 600°C overnight in a muffle furnace, the bone samples were manually crushed into a finely divided powder. Duplicate portions of each sample (1-5 mg each) were weighed to the nearest microgram and transferred to nonwettable plastic diffusion dishes (Falcon, Franklin Lakes, NJ) containing 3.0 mL of distilled water. The alkaline trap (50 μL of 0.05 N NaOH) was placed in three drops on the inside of the diffusion dish lid. The inside periphery of the lid was ringed with Vaseline and secured to the dish bottom. Through a hole previously burned in the lid with a soldering iron, 3.0 mL of 3.0 N sulfuric acid saturated with hexamethyldisiloxane were injected into the dish. The hole was immediately sealed with Vaseline. The dishes, including those containing the fluoride standards (100, 500, and 1,000 nmol prepared from NaF), were gently swirled on a rotary shaker overnight. The lids were then removed from the dish bottoms and the NaOH trap was buffered (pH 5) by adding 20 μL of 0.20 N acetic acid. The final volume was adjusted to 75 μL with distilled water using a fixed volume pipettor. The electrodes were placed in contact with this solution until a stable mV value was obtained (ca. 2 minutes).

In addition to the diffused standards, nondiffused standards were prepared using the same reagents. These standards were made to have the same concentrations as those of the diffused standards assuming that all the fluoride had been captured in the NaOH trap. Comparisons of the mV readings of the nondiffused and diffused standards showed that recovery of the diffused standards was virtually complete (recovery range, 98-103%). The bone ash fluoride concentrations are expressed as mg F/kg (or ppm).

Results

Type of Bone Specimen. For each of the five subjects, the measurement from the frozen unaffected bone specimen was

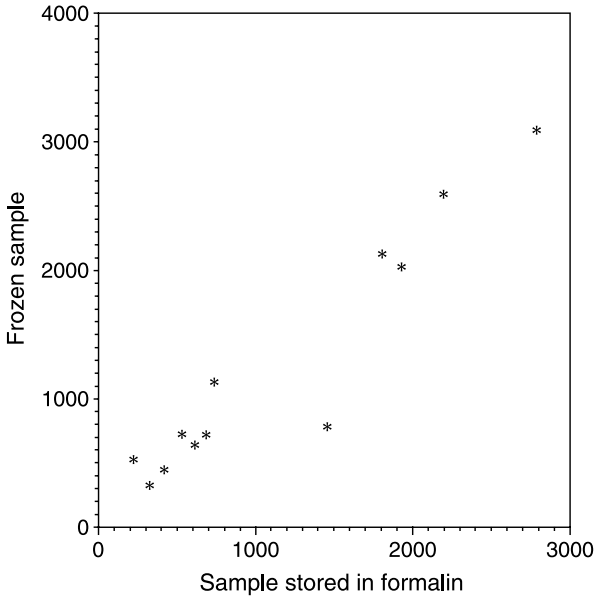

Figure 2. Fluoride measurement based on frozen samples compared with those based on samples stored in 10% formalin for 12 subjects.

Table 1. Estimated mean and CV with 95% confidence interval for each sample source

Source	Mean (mg F/kg)	CV (%)	95% Confidence interval
Deer low	118.70	4.7	2.6-6.8
Deer medium	734.22	4.0	1.4-6.6
Deer high	1,790.00	4.3	1.9-6.8
Human low	2,633.95	7.2	3.3-11.1
Human high	4,137.25	4.4	1.7-7.0

plotted against that from the frozen iliac crest specimen (Fig. 1). The mean measurement from the iliac crest samples was 1,648.4 mg F/kg, and the mean measurement for the frozen bone was slightly higher at 1,791.5 mg F/kg. Individual subject differences ranged from −851 to 1,239 mg F/kg, and the mean difference was +143.1 mg F/kg (P = 0.71). The Pearson correlation was 0.79 (P = 0.11), which suggests a positive linear correlation. The Spearman correlation of 0.90 (P = 0.037) suggests that the rankings were largely unchanged by source of the bone sample. A paired t test failed to show statistically significant differences between frozen unaffected and iliac crest bone samples; however, there were only five observations.

Method of Storage. In Fig. 2, the fluoride measurement based on a frozen sample was plotted against the measurement based on a sample stored in 10% formalin for each of 12 subjects. The measurement from the frozen sample was larger than the measurement using the sample stored in 10% formalin for 10 subjects. Differences ranged from −673 to +391 mg F/kg 1. However, the mean difference was not significant using a paired t test (+116.4 mg F/kg; P = 0.19; 95% confidence interval, 47.4-280.2). The Pearson and Spearman correlations were both >0.94 (P < 0.0001), a significant strong positive relationship. These results suggest that the method of storage does not have an important effect on fluoride measurements.

Measurement Reproducibility. Least-squares estimates were obtained for fixed and random effects in the mixed model (Eq. A) using data from the type of bone and method of storage experiments. The fixed effects were not significant (P > 0.25). The variance component estimates were σ_a^2 = 0.377 for subjects and σ^2 = 0.069 for error. The resulting estimate for the intraclass correlation was 0.85 (95% confidence interval, 0.71-0.98).

Assay Variability. For two human samples and three deer samples, there was sufficient material available. In Table 1, the estimated means, CVs, and confidence intervals are given. The variance component estimates for the CV were 4.3% using the deer samples, 5.9 using the human samples, and 5.0 (95% confidence interval, 3.74-6.26) using all samples. The mean fluoride levels of the deer samples were lower than those of the human samples available. However, when compared with the mean levels of the samples stored in formalin and used in the pilot, the high deer sample was well within that range.

Discussion

Iliac crest bone specimens were used to evaluate fluoride content in bone at a site distant from the tumor site. Correlations between normal bone near the affected area and normal iliac crest bone were strong and positive. Although we did not find statistically significant differences between bone near the tumor site and bone at the iliac crest, the power to detect such differences was limited because only five pairs were available.

The human bone samples collected were stored either in 10% formalin-fixed solution or as frozen specimens. Frozen bone samples contain cellular material, although sparse, that may be used in future genetic testing. Our results comparing these two forms of bone specimen storage yielded a strong positive correlation, and there was no evidence of a significant difference in means. Therefore, the results for this study suggested that the bone specimens stored in 10% formalin-fixed solution may be used in further projects studying the effects of fluoride in osteosarcoma cases.

Deer jaw bone is available in sufficient quantity to serve as a quality control. In this pilot, the fluoride levels reported for the deer jaw bone are similar to the human bone. The estimated CVs from deer jaw bone and human bone were both in the 4% to 7% range. Thus, the deer jaw bone, which is more readily available, could be used as quality control samples in further studies involving hexamethyldisiloxane-facilitated diffusion analysis of fluoride.

Epidemiologic studies planned to evaluate the association of fluoride in bone and the risk of cancer may require that samples be analyzed over a period of time. The day-to-day laboratory reproducibility of hexamethyldisiloxane-facilitated diffusion analysis of fluoride in human and deer jaw bone yielded acceptable results with average CVs below 10% and an overall estimate of 5%.

The ICC is the ratio of the biological variability among study subjects to the total variability, and in this situation, total variability included sources of variation associated with site of bone sampling and bone sample preparation as well as the lab assay procedures. The ICC is of importance because it indicates the effect of measurement error on study results. A regression analysis relating the log relative risk of disease to the log assay level will be attenuated by a factor equal to the ICC. Here, the estimated ICC is 0.86, and the estimated downward bias is only 14% so that the estimates of the relative risk will suffer little attenuation.

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